Chapter 4

A Transmembrane Single-Polypeptide-Chain (sc) Linker to Connect the Two G-Protein–Coupled Receptors in Tandem and the Design for an *In Vivo* Analysis of Their Allosteric Receptor-Receptor Interactions

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Abstract

A transmembrane (TM) single-polypeptide-chain (sc) linker can connect two G-protein–coupled receptors (GPCRs) in tandem. The priority of a gene-fusion strategy for any two class A GPCRs has been demonstrated. In the striatal function, dopamine (DA) plays a critical role. In the striatum, how the GPCR for adenosine, subtype A_{2A} ($A_{2A}R$), contributes to the DA neurotransmission in the "volume transmission"/dual-transmission model has been studied extensively. In addition to the fusion receptor, i.e., the prototype $scA_{2A}R/D_2R$ complex (the GPCR for DA, subtype D_2), several types were created and tested experimentally. To further elucidate this *in vivo*, we designed a new molecular tool, namely, the supermolecule $scA_{2A}R/D_2R$. Here, no experiments on its expression were done. However, the TM linker to connect the nonobligate dimer as the transient class A GPCR nanocluster that has not been identified at the cell surface membrane deserves discussion through $scA_{2A}R/D_2R$. Supramolecular designs, are experimentally testable and will be used to confirm *in vivo* the functions of the two GPCRs interactive in such a low specific signal to the nonspecific noise (S/N) ratio in the neurotransmission in the brain. The sc also has, at last, become straightforward in the field of GPCRs, similar to in the field of antibody.

Keywords: oligomerization, adenosine A_{2A} receptor, dopamine D_2 receptor, receptor allostery, fusion protein, striatum, supramolecular protein assembly

1. Introduction

Dopamine (DA) [1] plays a critical part in the function in the striatum of the basal ganglia [2, 3]. In striatal DA neurotransmission, how the G-protein–coupled receptor (GPCR) for adenosine, subtype A_{2A} ($A_{2A}R$) [4, 5], works in the "volume transmission"/dual-transmission model [6] was explored previously [7, 8]. Moreover, the prototype single-polypeptide-chain (sc) heterodimeric $A_{2A}R/D_2R$ complex (the GPCR for DA, subtype D_2 [9]) [10] (**Figures 1–3**), a fusion receptor, and several other types were created and tested experimentally [11]. Supermolecules were also designed, none of which were constructed or tested [12], while referring to a relationship between nanoscale surface curvature and surface-bound protein oligomerization [13–15]. Here, the transmembrane (TM) linker to connect the nonobligate dimer will be discussed based on $scA_{2A}R/D_2R$.

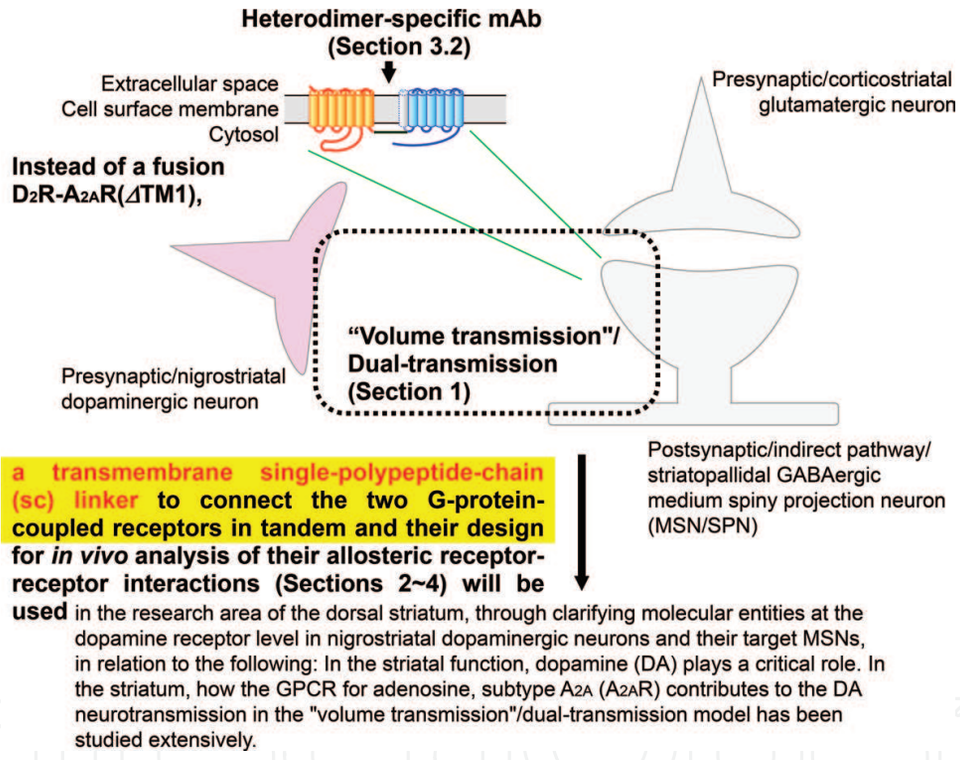

Figure 1. A transmembrane single-polypeptide-chain (sc) linker to connect two G-protein–coupled receptors in tandem. See text for details.

2. Making the single-polypeptide-chain to compensate the weak affinity of the two molecules

2.1. Glycine-glycine-glycine-glycine-serine (G_4S) linker

Approximately 30 years ago, a 15–amino acid linker [glycine-glycine-glycine-glycine-serine $(G_4S)_3$] was adopted to form a variable region fragment (Fv) analogue connected as a

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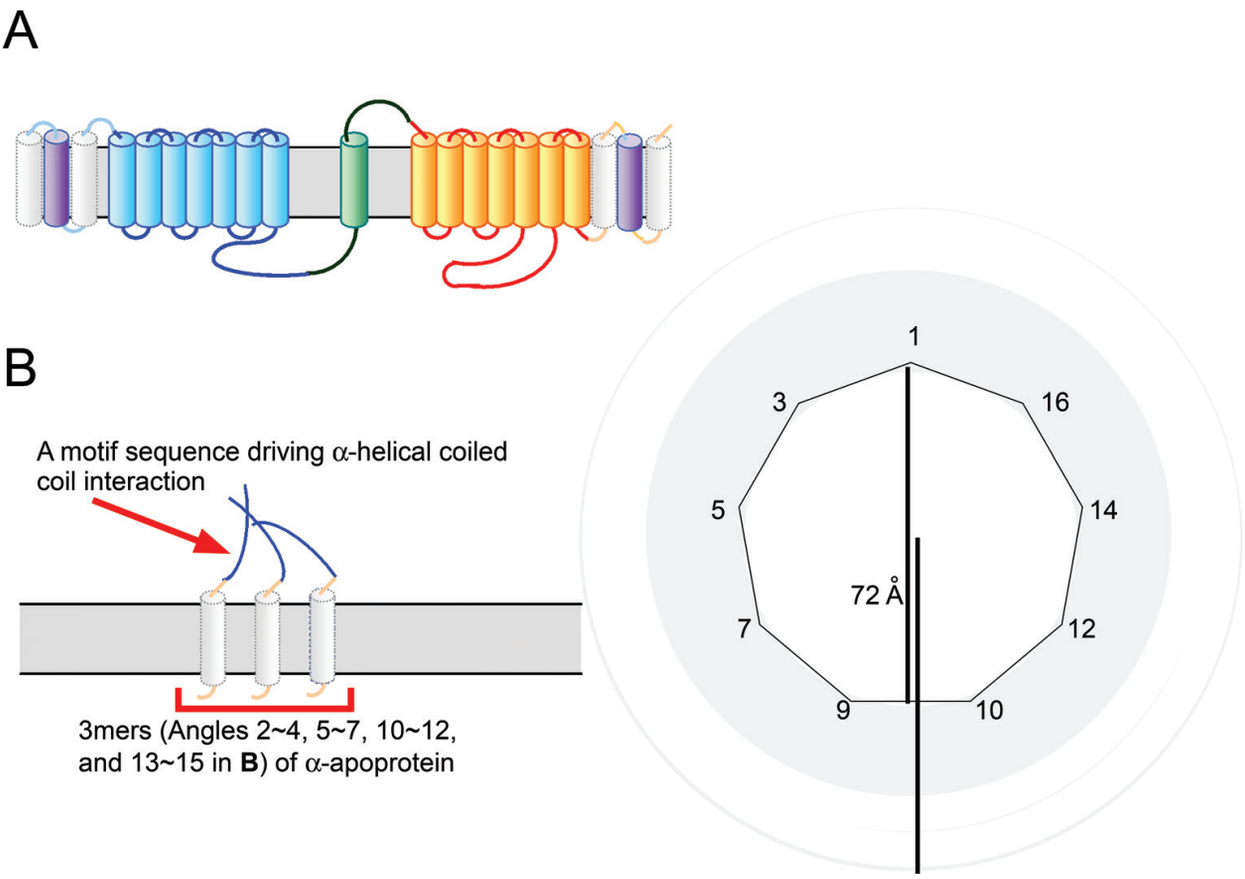

Figure 2. A supermolecule of an `exclusive´ dimeric GPCR with the oil-fence–like structure. (A) Using the light-harvesting antenna complex from *Thermochromatium tepidum*, the C-ter of an α-apoprotein (Angle 1 in B, in dotted line in gray) of two (Angles 1 and 16), each of which is concatenated in tandem through a CD4 transmembrane (TM) region in purple (in darker gray for a printed version in black and white) between Angles 1 and 16; CD4 is well known to make no formation of dimer itself, fused to the N-ter of TM helix 1 of the human prototype $scA_{2A}R/D_{2L}R$, i.e., $A_{2A}R$-*odr4*TM-$D_{2L}R$ colored (in the same gray as in Figure 1 for a printed version in black and white), and its C-ter of TM helix 7 fused to the N-ter of another α-apoprotein (Angle 8) of other two (Angles 8 and 9 here in B, as given angles of the complex, in dotted line in gray), i.e., the α-apoprotein-CD4TM-α-apoprotein-scGPCR-α-apoprotein-CD4TM-α-apoprotein fusion, is shown. Its expression as a fusion with the remaining ~12 α-apoproteins (presumed to be 16 mer originally in total), i.e., four 3mers (Angles 2–4, 5–7, 10–12, and 13–15 in B) of α-apoprotein fused to a motif sequence driving α-helical coiled coil interaction as lines in blue (in darker gray for a printed version in black and white) on the left in B because four 4mers and eight 2mers cannot exclude vacant supermolecules, could form a unified complex, with ~16 wild-type β-apoproteins (plus the translation initiation methionine) in total and pigments, thus surrounding $scA_{2A}R/D_{2L}R$. (B) The light-harvesting antenna complex (LH1, a gray circle) from *Thermochromatium tepidum* consists of 16 mer of the α-apoprotein [61 amino acids, with the intracellular N-terminus (N-ter); for clarity, instead of a hexadecagon (not shown here) with each angle numbered, some are shown as a nonagon] packed side by side to form a hollow cylinder of diameter 73–82 Å and the 16 helical β-apoproteins (46 amino acids plus the translation initiation methionine, with the intracellular N-ter) of an outer cylinder of diameter 96–105 Å, together with light-absorbing pigments (not shown here) 32 bacteriochlorophyll *a* (Bchl *a*) and 16 carotenoids (spirilloxanthin, Spx) [77]. Lines of 72 Å are shown in black. The figure art (A) is shown and was drawn and adapted from the published figure, i.e., the figure art (lower left) for $A_{2A}R$-*odr4*TM-$D_{2L}R$ in Fig. 1A (pp. 140), in our previous report [10], with written permission of the copyright owner, the Japan Society for Cell Biology. Only small but biologically important modifications were introduced.

single polypeptide chain (single-chain Fv), consisting of the heavy- and light-chain variable regions (V_H and V_L) of a monoclonal antibody (mAb). This was successfully produced in *Escherichia coli* or bacteriophage by protein engineering based on the crystallographic analysis of the antigen-binding fragments (Fabs) of antibodies, i.e., the carboxy-terminus

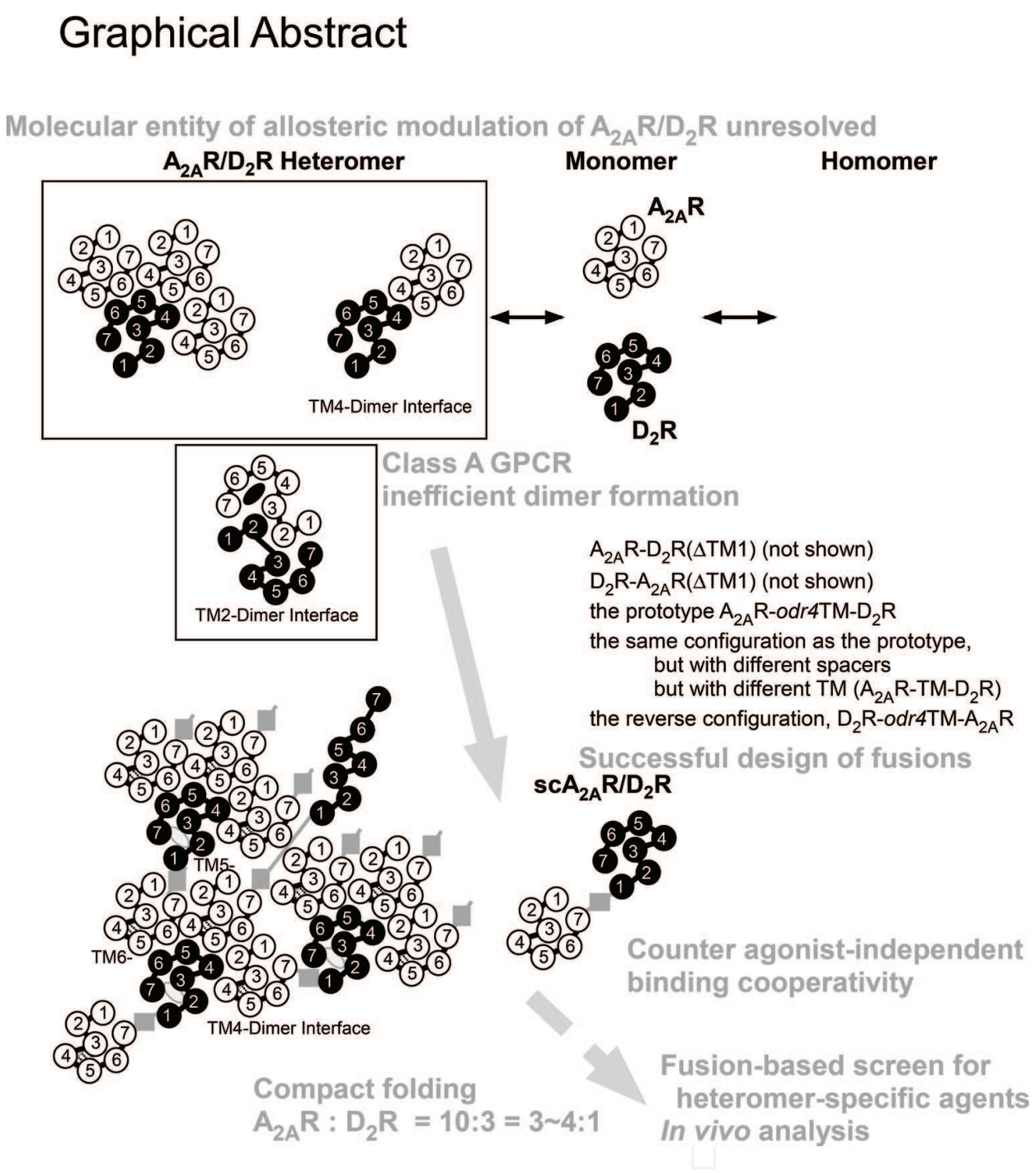

Figure 3. Transient class A GPCR nanocluster. TM helices (transverse sections) are shown as circles [numbered, in black (D_2R) or white ($A_{2A}R$)]. The $A_{2A}R$ and D_2R ligands are also shown as black and light gray ovals, respectively. *odr4*TM in the $scA_{2A}R/D_2R$ is shown in a gray rectangle. This figure is from the figure art of the graphical abstract in our previous report [11], with written permission of the copyright owner, Elsevier Inc.

(C-ter) of the V_H domain and the amino-terminus (N-ter) of the V_L domain, being at a distance of ≈3.5 nm [16, 17]. The use of a spacer (linker sequence) of ~30 amino acids, including G_4S, between the two proteins was established after that, giving $(G_4S)_3$: 3.5 nm [18]. As a compensation for the weak interactions between the two proteins, a tandem is especially

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useful. Under the molecular dynamics and the organizing principles of the plasma membrane [19], depending on the GPCR monomer-dimer dynamic equilibrium that is characterized by single-molecule imaging to date [20], the endocytosis of the GPCR is mediated by the clathrin-coated pit machinery [21]. The clathrin coats on the endosome vesicles resemble the architecture of a soccer ball, and each clathrin that forms a three-legged structure assembles into typical polyhedral cages, with an inscribed or circumscribed circle width diameter of approximately 25 nm ([11]: graphical abstract) (**Figure 3**). At issue are the tuning and amplitude of GPCR oligomerization. Using a β2V2R receptor chimera [the class A GPCR β_2 adrenergic receptor where the C-ter tail was exchanged for the class B GPCR vasopressin type 2 receptor (V2R) C-ter] [22], the existence, functionality, and architecture of internalized class B GPCR complexes, called supercomplexes or "megaplexes," resulting in sustained signaling, are reported to consist of a single GPCR, β-arrestin, and G protein. Additionally, GPCR-mediated extracellular signal-regulated kinase (ERK) activation is classified into two modes, including an early, β-arrestin–independent one, which may correspond to nanocluster activation at the cell surface membrane, and a late, β-arrestin–dependent one, which prolongs ERK activity. Nanoclusters are transient dynamic structures that are assembled by the lipid-anchored proteins [23]. With regard to Ras proteins, they are arrayed in nanoclusters comprising 6–8 proteins in domains that are 12–22 nm in diameter. However, the transient class A GPCR nanocluster has not been identified at the cell surface membrane.

2.2. Glycine-threonine (GT) linker

In a report by Twomey et al., in order to form the complex between the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-subtype ionotropic glutamate receptor GluA2 and the auxiliary protein stargazin (STZ), they used "a tandem construct, GluA2-STZ, where the N-ter of STZ was fused to the C-ter of GluA2 by a glycine-threonine (GT) linker."{[24]—(pp. 83, the right column—the second paragraph—line 1)}.

2.3. Glycine-serine-anchored 6– or 30–amino acid (GSxxGS) linker

In a report by Elegheert et al., they used the fusion protein of the $Cbln1_{C1q}$-$GluD2_{ATD}$. Cbln1 is a soluble synaptic organizer molecule with a compact jelly-roll β-sandwich fold and is a member of complement C1q-tumor necrosis factor superfamily that directly binds the ionotropic glutamate receptor δ2 extracellular N-ter domain GluD2 ATD. Cbln1 also interacts with presynaptic membrane-tethered neurexins that, with postsynaptic neuroligins, make up the transsynaptic bridges spanning the synaptic cleft [25]. Because of the weak affinity of the $Cbln1_{C1q}$-$GluD2_{ATD}$ interaction, they designed a construct that "linked a $Cbln1_{C1q}$ trimer with $GluD2_{ATD}$ into one continuous polypeptide chain by using short six-amino acid linkers (GSELGS and GSASGS in single-letter amino acid code, respectively)" and "by a 30-residue flexible Gly-Gly-Ser ($(G_2S)_{10}$) spacer, which may reach ~110 Å in length in a fully extended conformation and would allow quasi-unrestricted conformational sampling of the $GluD2_{ATD}$ by $Cbln1_{C1q}$-fused during the crystallization process." ([25]—supplementary text).

2.4. Transmembrane linker

Levitz et al. took advantage of a gene-fusion strategy used previously for microbial opsins [26] to construct a tandem dimer, where a TM linker connects the C-ter of the first copy of a class C GPCR, the metabotropic glutamate receptor mGluR2, to the N-ter of a second [27]. The gene-fusion strategy of Kleinlogel et al. proved to be useful in optogenetics and for any two class A GPCR rhodopsins, using "the light-activated microbial rhodopsins": Channelrhodopsin-2 (ChR2) derived from *Chlamydomonas reinhardtii* ["a cation-permeable channel that enables cell depolarization (neuronal activation) in response to blue light"] and halorhodopsin derived from *Natromonas pharaonis* (NphR or Halo) ["a chloride pump that enables cell hyperpolarization (neuronal silencing) in response to orange light"] [26], i.e., the intracellular C-ter of ChR2 through β helix (the 105-amino-acid N-ter fragment of the β subunit of the rat gastric H^+,K^+-ATPase) fused to the extracellular N-ter of NphR for the "precise co-localization and stoichiometric expression of two different light-gated membrane proteins." However, they found that in the fusion of ChR2(1-309) with βbR (a variant of the inhibitory proton pump bacteriorhodopsin from *Halobacterium salinarum*, containing an additional N-ter TM β helix), the insertion of an enhanced yellow fluorescent protein (EYFP) between the two proteins resulted in its functional expression (ChR2-EYFP-βbR). However, they did not try another β helix itself, the effects of their length, or the reverse type (such as bR-EYFP-βChR2), unlike the TM α helix linkers in our previous report [11]. Interestingly, rational *de novo* computational protein design of the α-helical domain is also reported [28–30].

The points raised in our study about how the $A_{2A}R$ contributes to the DA neurotransmission are addressed in a straightforward manner (**Figure 1**) (Section 3.1). Thus, making the single-polypeptide-chain has also, at last, become straightforward and is no longer an unusual approach to stimulate the weak affinity of two molecules in the field of neuroscience/GPCR, similar to in the field of immunology/monoclonal antibody. Additionally, "the glycan wedge" approach by "a 10-residue glycosylated linker" (ELSNGTDGAS in single-letter amino acid code) arranged between the ATD and ligand-binding domain (LBD) layers "in order to space them apart and disrupt potential mechanical ATD-LBD coupling" [25] is shown to function in inhibiting the association between protomers of the γ-aminobutyric acid (GABA) type B receptor ($GABA_B$)[$GABA_{B1}$ (GB1)]/[$GABA_{B2}$ (GB2)] heterodimer (via coiled-coil interaction of the cytoplasmic C-ter) but not that of the tetramer (GB2/GB1)-(GB1/GB2) [31].

Furthermore, the DA neurotransmission in the "volume transmission"/dual-transmission model could not physically adopt the supramolecular architectures, such as "the prototypical molecular bridge linking" postsynaptic GluD2 and the presynaptic neurexin, via Cbln1, besides a possible link between the glial $A_{2A}Rs$ and presynaptic or postsynaptic D_2R.

2.5. An enzyme-dependent covalent biotinylation occurs within 10–50 nm of the bait protein

BioID is an affinity purification approach where an *E. coli* BirA biotin-protein ligase, BirAR118G (BirA, with Gly replacing Arg 118), is fused to a bait protein expressed in cells and allows for the isolation and analysis of proximal proteins by streptavidin-based affinity

purification and mass spectrometry [32–36]. "Biotinylation by BioID is a mark of proximity and not evidence for physical interaction," and causing "the practical labeling radius of BioID *in vivo* to be ~10 nm" [32]. Thus, similar to the proximity ligation assay (PLA), BioID is a "proximity assay that may detect adjacent proteins that are not true interactor" [36]. Across the cell surface membrane, BirA-dependent covalent biotinylation cannot occur even within 10–50 nm of the bait protein.

3. The transmembrane-linked connection of the nonobligate dimer: $scA_{2A}R/D_2R$

3.1. An approach toward a class A GPCR dimer that is not fully formed

With a model of receptor-receptor interaction to regulate DAergic activity, a functional antagonistic interaction between $A_{2A}R$ and D_2R has been explicated [37–40]. Although allostery in a GPCR heterodimer is demonstrated [41–45], these class A GPCR dimers, unlike other class GPCRs that are fully formed [46], depend on the equilibrium between monomers and dimers [47]. This does not mean that such insufficient class A GPCR dimers or oligomers cannot function *in vivo*. Some types of protein-protein interactions, transient or weaker, "will be found to play an even more important role" in the cells [48, 49].

Interestingly, in the process of rational design and screening, we found that fusion of the two receptors stimulates the receptor dimer formation [10, 11]. In these studies, by fusing the cytoplasmic C-ter of the human brain–type $A_{2A}R$ (that is derived from Dr. Shine's cDNA [50]) ([12]: Fig. S1) through the TM domain of a type II TM protein (Section 4.3) with the extracellular N-ter of D_2R in tandem, we made successful designs for a fusion receptor, single-polypeptide-chain (sc) heterodimeric GPCR complex $A_{2A}R/D_2R$ [10, 11]. However, the resulting prototype $scA_{2A}R/D_{2L}R$ ($D_{2L}R$, the long form of D_2R) has a compact folding, i.e., a fixed stoichiometry (the apparent ratios of $A_{2A}R$ to D_2R binding sites), $A_{2A}R:D_2R = 10:3 = 3–4:1$ ([11]: graphical abstract) (**Figure 3**), and the $scA_{2A}R/D_2R$ expression system shows that the various designed types of functional $A_{2A}R/D_2R$ exist even in living cells, but there is no apparent allostery as a whole. Thus, to further clarify the heteromerization through $scA_{2A}R/D_{2L}R$, we tried to design other fusion proteins so as not to be formed/expressed as higher-order-oligomers, and we called these 'exclusive' monomers or dimers. First, we noted that GPCRs have general features of a TM helix 3 as the structural [at a tilt-angle of 35° to a perpendicular (vertical) line to the cell surface membrane plane]/functional hub and a TM helix 6 moving along 14 Å after activation [51] and of $A_{2A}R$, with a bundle width diameter of approximately 3.6 nm [12]. Thus, using a partner to increase spacing [25] without identifying and specifically blocking the interacting portions between the receptors, we created the designs for nonoligomerized 'exclusive' monomeric $A_{2A}R$ and/or D_2R in order to exclude their dimer/oligomer formation [12] (Sections 4.1.1 and 4.1.2), and did those for the 'exclusive' dimer [12] [Fig. 2, (Section 4.2)]. Such a self-assembled molecular architecture will entirely hold either a monomeric receptor or single dimeric $A_{2A}R/D_2R$ alone, but none of the oligomers. Although we constructed or tested none of these new fusions, we aimed to obtain heterodimer-specific agents using the

fusion receptor $scA_{2A}R/D_2R$. Indeed, we can take an example of a universal influenza vaccine that was engineered by fusing two polypeptides. The polypeptides originally resulted from the limited proteolysis of the native conformation of the hemagglutinin (HA), a trimeric membrane protein of influenza viruses, and their conformations were retained and stabilized in the vaccine engineering [52]. Such 'exclusive' forms of single-chain dimers are useful analytical tools, allowing us to address this point. Additionally, in the postsynaptic striatopallidal γ-aminobutyric acid (GABA)-ergic medium spiny projection neurons (the indirect pathway), expressing both $A_{2A}R$ and D_2R, an *in vivo* analysis of knock-in mice of the $scA_{2A}R/D_2R$ would elucidate the functional $A_{2A}R/D_2R$ with the antagonism [12], thus, to confirm us, *in vivo*, that such a low S/N ratio interaction between $A_{2A}R$ and $D_{2L}R$ functions in the DA neurotransmission in the striatum.

The heterodimeric interaction of $A_{2A}R$ with D_2R depends, in part, on the cytoplasmic C-ter region of $A_{2A}R$ [53]. Although the three-dimensional (3D) structures of the GPCR heterodimer also remains unresolved crystallographically [39], a possible monovalent agent acting on GPCR heterodimers was made reference to, and a screen needs developing to this end [54]. Our goal is to prove that the above-mentioned scGPCR-based screen is such a system. Here, we aim to obtain such heterodimer-specific agents [12], using a supramolecularly [55–57] designed fusion receptor, $scA_{2A}R/D_2R$, i.e., nonoligomerized 'exclusive' monomer (Section 4.1) and dimer (Section 4.2) of the receptors, and the above-mentioned *in vivo* analysis of the functional antagonistic $A_{2A}R/D_2R$. The possible occurrence of an unsuitable folding into a 3D structure, such that the resulting receptor exhibits lower or false activity, should be avoided. This is attributed to the interaction between a single 'exclusive' form of either the receptor monomer or $scA_{2A}R/D_2R$ and the surrounding fence-like architecture, while considering a single bond between two carbon atoms, with a C—C covalent bond with a distance of 1.5 Å (Section 4.3).

3.2. The molecular populations of the $A_{2A}R/D_{2L}R$ species in cell membranes

To illustrate this point, for clarity, let us consider epitopes generated only in heterodimeric $A_{2A}R/D_{2L}R$, but not in monomeric (and/or homodimeric) $A_{2A}R$ or $D_{2L}R$ [11] (**Figure 1**), which is in accordance with findings on the existence of agonistic/antagonistic (active/inactive-state-specific) or dimer-specific antibodies (nanobody) ([12]: Table 1). A broad and extremely potent human immunodeficiency virus (HIV)-specific mAb, termed 35O22, is reported, which binds the gp41−gp120 interface of the viral envelope glycoprotein (trimer of gp41–gp120 heterodimers) [58] ([12]: Table 1). This dimer-specific mAb was obtained despite not being immunized. The existence of virus-neutralizing mAbs, such as 35O22, which recognizes HIV-1 gp41–gp120 interface [58], and 2D22, locking the dimeric envelope proteins of dengue virus type 2 [59], is suggestive of that of the heterodimer-specific mAb that we are interested in. Additionally, transient nucleotide-bound β2-Gs species that are distinct from known structures are revealed [60]. Thus, the expression of homogeneous molecular species, either monomer or dimer, but not the mixture of both, followed by their membrane preparation appropriate to our needs, is necessary and worthy to be addressed experimentally.

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4. Architecture of a transmembrane-linked scA$_{2A}$R/D$_2$R

4.1. Supramolecular monomer

4.1.1. Protein assembly regulation and 'exclusive' monomers, supramolecularly designed using the Cε2 domain of IgE-Fc: the scA$_{2A}$R/D$_2$R-transmembrane linker makes both receptors stay away from each other

The molecular entity of the allosteric modulation of A$_{2A}$R/D$_2$R remains unresolved. To solve the insufficiency of the dimer formation of A$_{2A}$R/D$_{2L}$R, various scA$_{2A}$R/D$_2$R constructs, with spacers between the two receptors, were created (**Figure 3**). Successful designs of fusions, A$_{2A}$R-D$_2$R(*Δ*TM1) (not shown), D$_2$R-A$_{2A}$R(*Δ*TM1) (**Figure 1**), the prototype A$_{2A}$R-*odr4*TM-D$_2$R, fusions, which have the same configuration as the prototype, but with different spacers, and the same configuration as the prototype, but with different TM (A$_{2A}$R-TM-D$_2$R), and the reverse configuration, D$_2$R-*odr4*TM-A$_{2A}$R, were designed. Using whole cell binding assays, the constructs were examined for their binding activity. Two papers [61, 62] inspired us to also design the following fusions ([12]: Fig. 1B): first, the conversion between a single- and two-antigen binding brought by using a hinge/domain in the designed antibody was reported. Then, in models of viral fusogenic proteins, both steric hindrance and conformational changes, i.e., negative cooperativity, were referred to. Accordingly, we took advantage of the structure of the complete Fc fragment (Fc) of immunoglobulin (Ig) E, including the Cε2 domains, which is a compact, bent conformation ([63]: pp. 205, the right column-line 2 from the bottom; [64]) (the human IgE has a rigid Cε2 domain of Fc portion, instead of lacking a flexible hinge region, in contrast to other class/subclasses, such as IgG1. Upon binding of an allergen to the IgE that is already bound to the high-affinity receptor FcεRI, the antigen binding fragment (Fab) portion transduces it to the FcεRI [64]). Thus, the expression of the 'exclusive' monomeric GPCRs linked with the transmembrane plus human Cε2 domain (here in a loop), i.e., the C-ter of the *odr4*TM of the prototype scA$_{2A}$R/D$_{2L}$R fused to the N-ter of Cε2 and its C-ter fused to the N-ter of the D$_{2L}$R, could separate from each other. Whereas the prototype A$_{2A}$R-*odr4*TM-D$_2$R stimulates the dimerization of A$_{2A}$R and D$_2$R, this type of Cε2-intervening scA$_{2A}$R/D$_2$R makes both receptors push out each other, resulting in two 'exclusive' monomers. To this end, additional bulky molecules at both the N-ter and the C-ter of the fusion would be required.

4.1.2. Another `exclusive´ monomeric GPCR with the oil-fence–like structure: a supermolecule using transmembrane apoproteins from a bacterial light-harvesting antenna complex

The human A$_{2A}$R structure PDB 3EML [12] has a bundle width diameter of approximately 36 Å. It was determined by the T4-lysozyme fusion strategy [65], where most of the intracellular loop 3 (Leu209$^{5.70}$–Ala221$^{6.23}$: the Ballesteros-Weinstein numbering scheme is shown in superscript) [66] was replaced with a lysozyme from T4 bacteriophage, and the C-ter tail (Ala317–Ser412) was deleted to improve the likelihood of crystallization. According to recent papers, 'the most complex designed membrane proteins contain porphyrins that catalyze

transmembrane electron transfer' [55]. The peripheral light-harvesting antenna complex (LH2) [12] derived from the purple bacterium *Rhodopseudomonas acidophila* (*Rhodoblastus acidophilus*) strain 10050 is made up of both a 9 mer of the transmembrane α-apoprotein (53 amino acids, with the intracellular N-ter) grouped side by side to form a hollow cylinder with a radius of 18 Å and the 9 transmembrane helical β-apoproteins (41 amino acids, with the intracellular N-ter) of an outer cylinder with a radius of 34 Å, together with porphyrin-like, light-absorbing pigments bacteriochlorophyll *a* (Bchl *a*) and carotenoids [67]. Thus, a complex could be formed as an `exclusive´ monomeric GPCR with the oil-fence–like structure, by expressing both the C-ter of this α-apoprotein fused to the N-ter of TM helix 1 of GPCR, plus the C-ter of the TM helix 7 fused to the N-ter of another α-apoprotein, and other 7 wild-type α-apoproteins, together with 9 β-apoproteins and pigments [12].

4.2. An `exclusive´ dimeric GPCR with the oil-fence–like structure: a supramolecular dimer

Using the LH complex from *Thermochromatium tepidum*, the C-ter of the second copy (**Figure 2**: Angle 1 in B, in dotted line in gray) of two α-apoproteins (Angles 1 and 16), each of which is concatenated in tandem through the human leukocyte antigen (cluster of differentiation, CD) CD4 TM region (in purple between Angles 1 and 16; CD4 is well known to make no formation of a dimer itself), fused to the N-ter of TM helix 1 of the human prototype $scA_{2A}R/D_{2L}R$, i.e., $A_{2A}R$-*odr4*TM-$D_{2L}R$ (colored), and its C-ter of TM helix 7 fused to the N-ter of another α-apoprotein (Angle 8) of the other two (Angles 8 and 9 herein in B, as given Angles of the complex, in dotted line in gray), i.e., the α-apoprotein-CD4TM-α-apoprotein-scGPCR-α-apoprotein-CD4TM-α-apoprotein fusion, is shown. Its expression as a fusion, with the remaining ~12 α-apoproteins (presumed to be 16 mer originally in total), i.e., four 3mers (Angles 2–4, 5–7, 10–12, and 13–15 in B) of α-apoprotein fused to a motif sequence driving α-helical coiled coil interaction (as blue lines on the left in B) because four 4mers and eight 2mers cannot exclude vacant supermolecules, could form a unified complex, with ~16 wild-type β-apoproteins (plus the translation initiation methionine) in total and pigments, thus surrounding $scA_{2A}R/D_{2L}R$.

4.3. Predicting the interaction between the TM linker, *odr4*TM, in prototype $scA_{2A}R/D_2R$ and TMs of its surrounding (fence-like) LH proteins

Protein-protein interactions can be classified on the basis of their binding affinities [48, 49, 68, 69]: by definition, unlike permanent interactions with high affinities (K_d in the nM range), proteins interacting transiently, either weakly or strongly, show a fast bound-unbound equilibrium, with K_d values typically in the μM range or less. In our previous reports [10, 11], among the GPCR protein-protein interactions, such as the disulfide bond formation of the N-ter, coiled-coil interaction of the cytoplasmic C-ter, and TM interaction [70], a type II TM protein with a cytoplasmic N-ter segment, single TM, and extracellular C-ter tail, i.e., the *Caenorhabditis elegans* accessory protein of odorant receptor (*odr4*) [71], was first selected for a connection between the N-ter receptor half ($A_{2A}R$) and the C-ter receptor half ($D_{2L}R$) of the $scA_{2A}R/D_{2L}R$. Then, it was demonstrated that the insertion of some other TM sequence, instead

of the *odr4*TM sequence, works similarly or that it does not have to be *odr4*TM to work, using the $scA_{2A}R/D_{2L}R$ designed with another TM of a type II TM protein, the human low-affinity receptor for IgE designated CD23 (Section 3.1).

Among the Smart blast search of *odr4*TM hits, a hit of a photosynthetic protein [of photosystem (PS) II reaction center (RC)] is impressive to us because of the use of its surrounding apoproteins in the bacterial LH complex in the supramolecularly designed $scA_{2A}R/D_2R$, as described above [12]. The Blastp search of the *odr4*TM against all nonredundant GenBank databases and the human genome gave no hits for human CD23.

On the other hand, CD23 is highly conserved among mammals. The Smart blast search suggests that human CD23TM is selected as a preferable experimental design due to little relationship between the two protein TMs.

In photosynthesis, certain protein complexes, such as the PS (or the LH1 and the RC), build the highly efficient, light-induced charge separation across the membrane, followed by electron transport. More strictly, PSI forms supercomplexes with the RC and the light-harvesting proteins, i.e., PSI-type RC and LHCI, and PSII supercomplexes are PSII-type RC, core light-harvesting proteins (CP43 and CP47), and peripheral LHCII in aerobic photosynthesis (algae, cyanobacteria, and plants); PS is a type RC, core LH1, and peripheral LH2 in anaerobic photosynthesis (purple bacteria). Whether this quality of photosynthetic light reactions is negligible or not in our 'exclusive' supramolecular forms has not been tested. The rearrangement of each molecule in this architecture or exchange with similar but unrelated molecules [72] would be necessary to inhibit this completely and surround the prototype $scA_{2A}R/D_2R$.

In a recent report on PSII biogenesis, it is shown that, unlike its cyanobacterial counterpart, the PSII RC protein D1 C-terminal processing enzyme of a land plant *Arabidopsis* is essential for assembling functional PSII core complexes, dimers, and PSII supercomplexes [73], demonstrating a discrepancy in PSII protein assembly [72] between cyanobacterium and *Arabidopsis*. Thus, whether the LH is attached and expressed to form our supermolecules in animal cells requires testing. The D1 and D2 subunits of the PSII and the M and L subunits of the bacterial photosynthetic RC are members of the Photo RC (cl08220) protein superfamily. However, these proteins differ in their number of TM helices [12, 74–77]. In addition, for the following three reasons, it is persuasive to predict the interaction between *odr4*TM in prototype $scA_{2A}R/D_2R$ and TMs of its surrounding (fence-like) LH proteins: (1) based on findings that the RC affects the LH1 complex shape [78], (2) and that it remains unknown what structural features lead to the consequent differences in the nonameric and octameric apoprotein assemblies in LH2, respectively, from *Rhodopseudomonas acidophila* and another purple bacterium, *Rhodospirillum* (*Phaeospirillum*) *molischianum* [79], thus meaning that it remains unknown why LH1 surrounds the RC but LH2 does not, (3) and furthermore, due to the difficulty in membrane-protein topology prediction itself, up to 25% precision, "to predict interaction sites from sequence information only" {[80]: supporting information—[Table 9. List of PDB IDs of all monomers (Test-set 1)] (pp. 38, line 27), human $A_{2A}R$ (PDB: 3EML) ([12]: Fig. 2A) (Section 3.1.2) defined as a monomer}.

Thus, as a source of information for the prediction of the protein-protein interactions [81], multiple sequence alignments were analyzed between the *C. elegans odr4*TM or human CD23 TM and TMs of core RC proteins (L/M/H) of *T. tepidum* or *Rhodopseudomonas* (Blastochloris) *viridis* [12]. It indicates no relationship [12], suggesting that the αβ subunits for the LH surrounding $scA_{2A}R/D_2R$ do not affect their core GPCR itself substituted for the core RC, even if each protein in this supermolecule is assembled to form it. Whether alternative interactions between core GPCRs and surrounding LH subunits in our supermolecules exist remains unknown because our designs are only back-of-envelope sketches and also, 3D-models, especially of membrane proteins, have their limitations [12, 82–84].

5. Summary and outlook

The supramolecular designs of transmembrane-linked $scA_{2A}R/D_{2L}R$, 'exclusive' monomers and dimers using the Cε2 domain of IgE-Fc or apoproteins of the bacterial light-harvesting antenna complex, allowing us to express the class A GPCR by receptor protein assembly regulation, i.e., the selective monomer/nonobligate dimer formation, are experimentally testable and will be used to confirm, *in vivo*, that such low S/N ratio interaction between $A_{2A}R$ and $D_{2L}R$ functions in the dopamine neurotransmission in the striatum.

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Conflicts of interest

The authors declare no competing financial interests.

The founding sponsors had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, and in the decision to publish the results.

Notes

Kinki University, to which Takashi Masuko has been affiliated, changed its English name to Kindai University in the year 2016 (URL: http://www.kindai.ac.jp/english/about/history.html).

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Author contributions

T.K. contributed to the planning and interpretation of all of the experiments and conducted all of them and performed the design [at The University of Tokyo General Library (Hongo, Bunkyo-ku, Tokyo)] [from April 1, 2013 to date (this submission)] of vectors for the nonoligomerized 'exclusive' monomer and dimer, experiments such as antibody screening at TMIN [11], the *in silico* search and wrote the paper; O.S. contributed to the interpretation of all of the experiments done at TMIN [11]; T.M. performed immunization and cell fusion to make hybridomas at Kinki University and, with T.K., wrote the paper; H.O. performed the design (It started from December 2014) of the vectors for the *in vivo* analysis and, with T.K., wrote the paper; K.F. supervised the project of the electrophysiological studies (It started from November 2007) [11]; with T.K., D.O.B.-E. analyzed the $A_{2A}R$ SNP and wrote the paper; and H.N. supervised the overall project by the end of March 2007 and also contributed to the interpretation of all of the experiments by the end of March 2007. All the authors commented on the manuscript, except for H.N., who is, at present, under a departure from research activity.

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